VERIFICATION OF A TRANSLATION

I, the below named Translator, hereby declare that;

I am the translator of the Japanese specification of U.S. Patent Application under Title of the Invention " A METHOD FOR IMPROVING PLANT TRANSFORMATION EFFICIENCY BY ADDING COPPER ION" filed on the 10th day of February 2006.
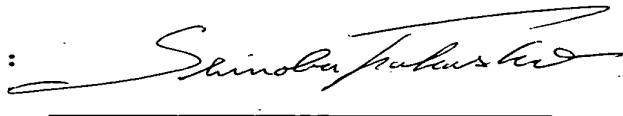
That I believe the attached English translation is a true and complete translation of said application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 23rd day of August 2006.

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    Attorney (Name)   Mr. Ichio SHAMOTO
    Submitted date   August 12, 2004

Received the following document

No.	Document	Arrangement No.	Receipt No.	Filing Receipt (indication of case)
1	Patent Application	YCT-960	50401363913	PCT/JP2004/011599

SPECIFICATION

A METHOD FOR IMPROVING PLANT TRANSFORMATION
EFFICIENCY BY ADDING COPPER ION

5   TECHNICAL FIELD

[0001]   The present invention relates to an efficient method for Agrobacterium-mediated gene introduction into plant materials.

10   BACKGROUND ART

[0002]   Agrobacterium-mediated gene introduction is a method for transforming plants through the use of a function of Agrobacterium.   A soil bacterium Agrobacterium (*Agrobacterium tumefaciens*) functions in such a manner that the T-DNA forming a part of its Ti (tumor-inducing) plasmid involved in the pathogenicity of the Agrobacterium is integrated into the genome of a plant when it infects the plant.   Agrobacterium-mediated plant transformation is a method for introducing a desired gene into the genome of a plant through the use of the function of Agrobacterium described above by constructing a transforming plasmid in which the T-DNA region of the Ti plasmid is replaced by the gene desired to be introduced into the plant genome and then using Agrobacterium prepared to carry the transforming plasmid in place of the Ti plasmid.

[0003]   Agrobacterium-mediated plant transformation was originally developed mainly as a method for transforming dicotyledons because Agrobacterium were

thought to infect only dicotyledons but not monocotyledons. Subsequently, various attempts for Agrobacterium-mediated gene introduction into monocotyledons were also made, and super-binary vectors having a part of the virulent genes of super-virulent Agrobacterium strains were developed and reported to be useful for stably transforming even monocotyledons such as rice and maize with relatively high efficiency (e.g., see Japanese Patent No. 2,649,287; Japanese Patent No. 3,329,819; Hiei, Y., et al., (1994), The Plant Journal, Vol. 6, p. 271-282; and Ishida, Y., et al., (1996), Nature Biotechnology, Vol. 4, p. 745-750). Successful examples of Agrobacterium-mediated transformation of monocotyledons such as wheat, barley and sorghum were also reported (e.g., see Cheng, M., et al., (1997), Plant Physiol., Vol. 115, p. 971-980; Tingay, S., et al., (1997), Plant J., Vol. 11, p. 1369-1376; and Zhao, Z-Y., et al., (2000), Plant Mol. Biol., Vol. 44, p. 789-798), and Agrobacterium-mediated transformation also began to be widely applied to monocotyledons.

[0004] Agrobacterium-mediated transformation generally has many advantageous features such as high efficiency, low copy number transgenes, transducibility of such a specific region as T-DNA without being fragmented, and short-term culture for obtaining transformants resulting in less mutation during culture. Thus, it is now widely used as the most useful means for transforming many plant species irrespective of whether they are dicotyledonous or monocotyledonous.

[0005] Agrobacterium-mediated transformation is equally performed in all plants by contacting a material tissue with an Agrobacterium suspension, coculturing them and then selecting a transformed cell to produce a

5   transformed plant, though the materials and the compositions of the culture media vary with plant species. Generally, the plant tissue used as a material is infected with Agrobacterium without any special treatment except for optional sterilization (e.g., see Rogers, S. G., et al.,

10 (1988), Method for Plant Molecular Biology, p. 423-436, CA: Academic Press Inc.; Visser, R. G. F., (1991), Plant Tissue Culture Manual, B5:1-9, Kluwer Academic Publishers; McCormick, S., (1991), Plant Tissue Culture Manual, B6:1-9, Kluwer Academic Publishers; and Lindsey, K., et al.,

15 (1991), Plant Tissue Culture Manual, B7:1-13, Kluwer Academic Publishers).

[0006] Agrobacterium-mediated transformation has been reported for many plant species, but has the disadvantage that the transformation efficiency widely varies with plant

20 species, genotypes and material tissues (e.g., see Potrykus, I., et al., (1998), Agricultural Biotechnology, NY: Mercel Dekker Inc., p. 119-159). It is important to develop a technology enabling transformed plants to be stably obtained with high efficiency throughout the year

25 because many transformed plants must be produced when a cultivar containing a practical gene is to be bred. Moreover, transformation methods independent of plant species and genotypes would be very useful for efficiently

breeding practical cultivars. Development of transformation methods independent of material plant tissues would also be required for efficient transformation.

[0007] Thus, it is important to develop a method capable of improving gene transduction efficiency or transforming even plant species or genotypes involving difficulty in gene transduction. Many techniques for efficiently obtaining transformed plants have already been reported in various aspects such as the adaptation of the compositions of culture media, the alteration of marker genes, or promoters or the investigation of materials and treatment methods for materials. For example, treatment methods for materials by injuring tissues to improve infection efficiency or by centrifuging (e.g., see International Publication No. WO02/12520; Japanese Patent Public Disclosure No. 2000-342256) or heating (e.g., see Japanese Patent Public Disclosure No.2000-342255; Japanese Patent Public Disclosure No. 2000-342253) plant tissues without injuring them have been reported. The present inventors previously found that pressurization of plant tissues is useful for improving gene transduction efficiency (with the results unpublished).

[0008] Among monocotyledons, maize had the disadvantage that the Agrobacterium-mediated transformation efficiency is lower than that of rice. Various attempts have already been made to improve the Agrobacterium-mediated transformation efficiency of maize (e.g., see

Negrotto, D., et al., (2000), Plant Cell Reports, Vol. 19,
p. 798-803; Zhao, Z-Y., et al., (2001), Mol. Breed.,
Vol. 8, p. 323-333; Frame, B. R., et al., (2002), Plant
Physiol., Vol. 129, p. 13-22; and , Ishida, Y., et al.,
(2003), Plant Biotechnology, Vol. 14, p. 57-66). Various
previous attempts to improve the Agrobacterium-mediated
transformation efficiency of maize include selecting
transformed cells on N6 basal medium (e.g., see Zhao, Z-Y.,
et al., (2001), Mol. Breed., Vol. 8, p. 323-333), adding
silver nitrate and carbenicillin to culture media (e.g.,
see Zhao, Z-Y., et al., (2001), Mol. Breed., Vol. 8,
p. 323-333; and , Ishida, Y., et al., (2003), Plant
Biotechnology, Vol. 14, p. 57-66), adding cysteine to
coculture media (e.g., see Frame, B. R., et al., (2002),
Plant Physiol., Vol. 129, p. 13-22), etc., but the
resulting effects are still low. Transformation methods
with higher transformation efficiency would be desirable
especially for major crops associated with low
transformation efficiency such as maize not only when
practical transformed plants are to be produced but also
when the effect of a novel gene is to be tested.

[0009] Copper sulfate is contained as a minor salt in
a wide variety of media. Normally, the concentration of
copper sulfate in plant tissue culture media is 0.1 μM.
Recent report shows that various effects were observed when
adding copper sulfate at 50-fold to 500-fold higher
concentrations than normal levels to media in tissue
cultures and transformation tests of monocotyledons.

Ghaemi et al. (see Ghaemi, M., et al., (1994), Plant Cell, Tissue and Organ Culture, Vol. 36, p. 355-359) reported that embryoid formation increases by culturing anthers of wheat in a medium containing 10 mg/l copper sulfate and

5   2.5-5 mg/l silver nitrate. Zhang et al. (see Zhang, S., et al., (1999), Plant Cell Reports, Vol. 18, p. 959-966) reported that the induction ratio of shoot meristematic cultures (SMCs) increases by culturing shoots having emerged from ripe seeds of barley in a medium containing

10   5 µM copper sulfate and 30 g/l maltose. They describe that maltose is effective for decreasing brown tissue and that copper sulfate is effective for promoting shoot growth when shoot meristematic cultures are induced. It was also reported that the regeneration ratio or the number of

15   regenerated plants per callus increases in calli obtained by culturing immature embryos of barley (e.g., see Dahleen, L. S., (1995), Plant Cell, Tissue and Organ Culture, Vol. 43, p. 267-269; and, Cho, M-J., et al., (1998), Plant Science, Vol. 138, p. 229-244) and rice (e.g., see

20   Sahrawat, A. K. and Chand, S., (1999), J. Plant Physiol., Vol. 154, p. 517-522) in media containing copper sulfate. It was also reported that green tissues having regeneration potential induced in media containing copper sulfate are suitable as materials for transformation (e.g., see Visser,

25   R. G. F., (1991), Plant Tissue Culture Manual, B5:1-9, Kluwer Academic Publishers; and, McCormick, S., (1991), Plant Tissue Culture Manual, B6:1-9, Kluwer Academic Publishers).

[0010] Ishida et al. (see Ishida, Y., et al., (2003), Plant Biotechnology, Vol. 14, p. 57-66) investigated callus formation from immature embryos by culturing immature embryos of maize (cultivar: H99) inoculated and co-cultured

5   with Agrobacterium, on media containing 1-100 μM copper sulfate.   The callus formation improved in media containing 1-10 μM copper sulfate, but slightly.

[0011] As described above, previous reports showed that various effects were observed in tissue cultures of

10   monocotyledons by adding high concentrations of copper sulfate to media.   However, there has been no report on the effects of adding a metal salt containing copper ion on gene introduction efficiency and/or transformation efficiency.

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REFERENCES:

Patent document 1: Japanese Patent No. 2,649,287.

Patent document 2: Japanese Patent No. 3,329,819.

Patent document 3: International Publication No. WO

20   02/12520.

Patent document 4: Japanese Patent Public Disclosure No. 2000-342256.

Patent document 5: Japanese Patent Public Disclosure No. 2000-342255.

25   Patent document 6: Japanese Patent Public Disclosure No. 2000-342253.

Patent document 7: U.S. Patent No. 6,235,529.

Patent document 8: U.S. Patent No. 6,541,257.

Patent document 9: International Publication No. WO 95/06722.

Non-patent document 1: Hiei, Y., et al., (1994), The Plant Journal, Vol.6, p.271-282.

Non-patent document 2: Ishida, Y., et al., (1996), Nature Biotechnology, Vol.4, p.745-750.

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Non-patent document 13: Frame, B.R., et al., (2002), Plant Physiol., Vol.129, p.13-22.

Non-patent document 14: Ishida, Y., et al., (2003), Plant Biotechnology, Vol.14, p.57-66.

Non-patent document 15: Ghaemi, M., et al., (1994), Plant Cell, Tissue and Organ Culture, Vol.36, p.355-359.

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Non-patent document 20: Trick, H.N. and Finer, J.J., (1997), Transgenic Research, Vol.6, p.329-336.

Non-patent document 21: Amoah, B., et al., (2001), Journal of Experimental Botany, Vol.52, P.1135-1142.

Non-patent document 22: Hoekema, A., et al., (1983), Nature, Vol.303, p.179-180.

Non-patent document 23: Komari, T. and Kubo T., (1999), Methods of Genetic Transformation: Agrobacterium tumefaciens. In Vasil, I.K. (ed.) Molecular improvement of cereal crops., Kluwer Academic Publishers, Dordrecht, p.43-82.

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0012] An object of the present invention is to develop a method by which gene introduction is achieved with higher gene introduction efficiency than obtained by conventional methods for Agrobacterium-mediated gene introduction into plants. Another object of the present invention is to develop a method by which transformed cells are grown with higher growth efficiency than obtained from plant tissues by conventional methods for Agrobacterium-mediated gene introduction into plants. Still another object of the present invention is to develop a process for preparing a transformed plant using any of these methods.

MEANS FOR SOLVING THE PROBLEMS

[0013] As a result of careful studies to attain the objects described above, the present inventors found that stable and highly efficient gene introduction is achieved and stable and highly efficient cell growth is observed from the gene-introduced tissues by Agrobacterium-mediated gene introduction into plants using a medium enriched in a metal salt as compared with cases where a medium containing the metal salt at a normal level is used. The present inventors also found that stable and more highly efficient gene introduction is achieved by heating/centrifuging plant materials before they are infected with Agrobacterium in addition to gene introduction using a medium enriched in a metal salt. The present inventors also found that plant materials which gene introduction is carried out by using a medium enriched in a metal salt dramatically improve

transformation efficiency by selecting transformed cells from the gene-introduced plant materials as compared with cases where a medium containing the metal salt at a normal level is used.

[0014] Accordingly, the present invention relates to a method for Agrobacterium-mediated gene introduction into a plant material, comprising:

1) treating the plant material, and

2) infecting the plant material with an Agrobacterium, characterized in that a medium enriched in a metal salt containing copper ion is used in step 1) and/or 2).

[0015] In the method of the present invention, the metal salt present at a high concentration in the medium is a metal salt containing copper ion. A preferred metal salt used in the present invention is copper sulfate or copper gluconate, most preferably copper sulfate. Copper sulfate is not limited to either anhydrous or hydrated salt.

[0016] In the method of the present invention, the medium enriched in a metal salt means a medium containing a metal salt at a high concentration relative to the concentrations of the metal salt in basal media well known to those skilled in the art such as N6 basal medium, MS (LS) basal medium, B5 basal medium, NN basal medium, NT basal medium, Kao's basal medium, White's basal medium, etc. The high concentration means a concentration higher than the concentrations of the metal salt in the basal media.

[0017] Specifically, a medium prepared on the basis

of a basal medium such as N6 basal medium containing

0 mg/l, MS (LS) basal medium containing 0.025 mg/l, B5

basal medium containing 0.025 mg/l, NN basal medium

containing 0.025 mg/l, NT basal medium containing 0.025

5   mg/l, Kao's basal medium containing 0.025 mg/l, or White's

basal medium containing 0 mg/l $CuSO_4 \cdot 5H_2O$ and containing a

higher concentration of copper sulfate than the copper

sulfate concentration of the basal medium is a medium

enriched in a metal salt.

10   [0018]   As used herein, the metal salt containing

copper ion means a metal salt containing copper ion

normally absent or contained in minor amounts in the basal

media listed above.

[0019]   Examples of preferred concentrations are

15   1-100 µM, preferably 1-50 µM, more preferably 1-10 µM

copper sulfate and copper gluconate.

[0020]   In the method of the present invention, the

step in which a medium enriched in a metal salt is used is

any one of step 1) of preparing the plant material, and/or

20   step 2) of infecting the plant material with an

Agrobacterium.   Preferably, a medium enriched in a metal

salt is used in at least step 2) of infecting the plant

material with an Agrobacterium.   More preferably, a medium

enriched in a metal salt is used during at least the

25   co-culture stage included in step 2) of infecting the plant

material with an Agrobacterium.

[0021]   The method of the present invention may

further comprise subjecting the plant material to at least

one treatment selected from the group consisting of pressurization, heat treatment, centrifugation and sonication in step 1) of preparing the plant material and/or step 2) of infecting the plant material with an

5 Agrobacterium. Pressurization of the plant material is performed in liquid media at 1.7-10 atm for 0.1 second to 4 hours, preferably 2.4-8 atm for 1 second to 30 minutes. Heat treatment of the plant material can be performed by the methods described in various documents (Japanese Patent

10 Public Disclosure No.2000-342255; and Japanese Patent Public Disclosure No.2000-342253), e.g., at 33-60°C for 5 seconds to 24 hours, preferably 46 °C for 3 minutes. Centrifugation of the plant material can be performed by the method of Hiei et al. (International Publication No.

15 WO02/12520; and Japanese Patent Public Disclosure No. 2000-342256), e.g., at 100 G - 250,000 G for 1 second to 4 hours, preferably 20,000 G for 10 minutes. Sonication can be performed by the methods described in documents (e.g., Trick, H. N. and Finer, J. J., (1997), Transgenic

20 Research, Vol.6, p.329-336; and Amoah, B., et al., (2001), Journal of Experimental Botany, Vol.52, p.1135-1142). Any one of these treatments such as pressurization, heat treatment, centrifugation and sonication may be performed or any combination may be performed.

25 [0022] The method of the present invention may further comprise the steps of:

3) selecting a transformed cell, and

4) optionally regenerating the selected transformant,

subsequent to step 2) of infecting the plant material with an Agrobacterium.

[0023]   The method of the present invention may further comprise the steps of:

3) selecting a transformed cell, and

4) optionally regenerating the selected transformant, subsequent to step 2) of infecting the plant material with an Agrobacterium, wherein a medium enriched in a metal salt containing copper ion is used in at least one of the steps above.

[0024]   The gene introduction method of the present invention improves gene introduction efficiency as well as transformation efficiency, with the result that transformed plants can be efficiently obtained. Thus, the present invention also relates to a process for preparing a transformed plant characterized in that a gene introduction method of the present invention is used.

[0025]   The present inventors also found that the growth of regenerated plants is promoted by regenerating transformed plant materials using a medium enriched in a metal salt containing copper ion as compared with cases where a medium containing the metal salt containing copper ion at a normal level is used.

[0026]   Thus, the present invention also relates to a process for preparing a transformed plant by Agrobacterium-mediated transformation of a plant material, comprising:

1) preparing a plant material,

2) infecting the plant material with an Agrobacterium,

3) selecting a transformed cell, and

4) regenerating the selected transformant,

characterized in that a medium enriched in a metal salt

containing copper ion is used in step 4).

[0027]   Alternatively, the present invention relates

to a method for promoting the growth of a regenerated plant

characterized in that a medium enriched in a metal salt

containing copper ion is used in the step of regenerating a

plant from a dedifferentiated plant cell. The regenerated

plant cell here may or may not be a transformed cell, and

when it is a transformed cell, it may or may not be

transformed by an Agrobacterium-mediated method.

Methods for Agrobacterium-mediated gene introduction
and transformation

Agrobacterium-mediated gene introduction typically

comprises the steps of:

a) preparing a plant material;

b) preparing an Agrobacterium carrying a vector containing

a desired transgene; and

c) infecting the plant material prepared in step a) with

the Agrobacterium prepared in step b).

[0028]   In order to obtain a transformant, step c) may

be further followed by the steps of:

d) selecting a transformed cell; and

e) optionally regenerating the selected transformant.

[0029]   Specifically, for monocotyledons, it is

possible to use a method characterized in that the plant

material is cultured in a medium containing auxin (e.g.,
2,4-D (2,4-dichlorophenoxyacetic acid)) or cytokinin or the
like to turn it into a dedifferentiated state or a state
under dedifferentiation in step a) and infected with
Agrobacterium in step c) as described in a document
(Japanese Patent No. 2,649,287); or a method characterized
in that an immature embryo of the plant is used as the
plant material and cultured in a medium containing auxin
(e.g., 2,4-D) or cytokinin or the like in step c) without
dedifferentiating it in step a) as described in a document
(Japanese Patent No. 3,329,819).

[0030] Step a)

The "plant" used herein for gene introduction means to
include both monocotyledons and dicotyledons.
Monocotyledons include, but are not limited to, rice,
maize, barley, wheat, asparagus, sorghum and the like.
Dicotyledons include, but not limited to, tobacco, soybean,
potato, cotton, sunflower and the like. Preferably, the
plant is a monocotyledon, most preferably maize.

[0031] The "plant material" encompasses all the
aspects of plants to be used for Agrobacterium-mediated
transformation of plants including, but not limited to,
plant cells, leaves, roots, stems, fruits, plant tissues of
any other parts, immature embryos, calli or adventitious
embryo-like tissues (hereinafter referred to as calli or
the like, or simply calli), or whole plants.

[0032] A desirable plant form used in the methods of
the present invention is an immature embryo or a callus,

most desirably an immature embryo. As used herein, the expressions of plant cell, tissue and whole plant have the same meanings as commonly used in the art. As used herein, the immature embryo means the embryo of an immature seed

5   under maturation after pollination. The stage (maturation phase) of the immature embryo used in the methods of the present invention are not specifically limited, and it may be collected at any stage after pollination. However, it is preferably at a post-pollination stage of two days or

10   more. Preferably, the scutellum of an immature embryo capable of inducing a callus that can be dedifferentiated to regenerate a normal plant by the method described below after the transformation described below is used. The immature embryo is preferably an immature embryo of an

15   inbred line, F1 between inbred lines, F1 between an inbred line and an open-pollinated cultivar, or a commercially available F1 cultivar. As used herein, a callus means an undifferentiated cell clump under uncontrolled growth. A callus can be obtained by culturing a differentiated cell

20   of a plant tissue in a medium containing a plant growth regulator such as auxin (e.g., 2,4-D) or cytokinin (referred to as dedifferentiation medium). The treatment for obtaining a callus is called dedifferentiation treatment and this process is called dedifferentiation

25   process.

[0033] In step a), a material suitable for transformation is prepared by extracting a plant tissue, immature embryo or the like as appropriate from a plant,

seed or the like. Optionally, the plant material may be cultured before it is infected with an Agrobacterium.

[0034] The present invention is characterized in that a medium enriched in a metal salt containing copper ion is used during the process of preparing a plant material in step a), and/or during the process of infecting the plant material with an Agrobacterium in step c). Additionally, pressurization may be performed during the process of preparing a plant material in step a).

[0035] Step b)

A soil bacterium Agrobacterium (*Agrobacterium tumefaciens*) has long been known to induce crown gall disease in many dicotyledons, and in 1970s, it was discovered that its Ti plasmid is involved in pathogenicity and that the T-DNA forming a part of the Ti plasmid is integrated into plant genomes. Subsequently, it was shown that the T-DNA contains genes involved in the synthesis of hormones necessary for inducing cancers (cytokinin and auxin) and that these genes are expressed in plants though they are bacterial genes. Excision of the T-DNA and its transfer to plants require genes existing in the virulence region (vir region) on the Ti plasmid, and excision of the T-DNA requires border sequences flanking the T-DNA. Another Agrobacterium, *Agrobacterium rhizogenes* has a similar system based on its Ri plasmid (e.g., Figures 3 and 4 of Japanese Patent Public Disclosure No. 2000-342256).

[0036] A desired gene was expected to be integrated into plant genomes by inserting it onto the T-DNA because

the T-DNA is integrated into plant genomes by infection
with Agrobacterium. However, it was difficult to insert a
gene onto the T-DNA on the Ti plasmid by standard genetic
engineering techniques because the Ti plasmid is as large
5   as 190 kb or more. Thus, a method for inserting an
exogenous gene onto the T-DNA was developed.

[0037] Initially, disarmed strains in which the
hormone-synthesizing genes have been removed from the T-DNA
of the tumor-inducing Ti plasmid such as LBA4404 (see
10   Hoekema, A., et al., (1983), Nature, Vol. 303, p. 179-180),
C58C1 (pGV3850), and GV3Ti11SE were prepared. Two methods
were developed for introducing a desired gene into the
T-DNA of the Ti plasmid of Agrobacterium or introducing the
T-DNA carrying a desired gene into Agrobacterium using
15   these strains. The first method is called the intermediate
vector method wherein an intermediate vector that can be
easily genetically manipulated to insert a desired gene and
that can be replicated in E.coli is inserted into the T-DNA
region of a disarmed Ti plasmid of Agrobacterium by
20   homologous recombination via triparental mating.

[0038] The second method is called the binary vector
method based on the finding that the vir region is required
for integration of the T-DNA into plants but need not be
present on the same plasmid to serve its functions. There
25   exist virA, virB, virC, virD, virE and virG in the vir
region (Dictionary of Plant Biotechnology, published by
Enterprise (1989)), and the vir region refers to a region
containing all of these virA, virB, virC, virD, virE and

virG. Thus, a binary vector which is a small plasmid replicable in both Agrobacterium and E.coli into which the T-DNA is integrated, is introduced into Agrobacterium having a disarmed Ti plasmid.

[0039] Introduction of a binary vector into Agrobacterium can be performed by known methods such as electroporation and triparental mating. Binary vectors include pBIN19, pBI121, pGA482, etc., and many novel binary vectors based on them were constructed and used for transformation. In the Ri plasmid system, similar vectors were constructed and used for transformation.

[0040] Agrobacterium A281 is a super-virulent strain that has a wide host range and higher transformation efficiency than those of other strains. This characteristic is attributed to pTiBo542 of the Ti plasmid carried by A281. Two novel systems were developed using pTiBo542 until now. One uses EHA101 and EHA105 strains carrying a disarmed Ti plasmid of pTiBo542 and finds applications in transformation of various plants as a system having a high transforming ability by applying these strains to the binary vector system described above.

[0041] The other is a 'super-binary' vector (see Hiei, Y., et al., (1994), The Plant Journal, Vol. 6, p. 271-282; Ishida, Y., et al., (1996), Nature Biotechnology, Vol.4, p.745-750; Komari, T. and Kubo T., (1999), Methods of Genetic Transformation: Agrobacterium tumefaciens. In Vasil, I. K. (ed.) Molecular improvement of cereal crops., Kluwer Academic Publishers, Dordrecht, p.43-82; and

International Publication No. WO 95/06722) system (e.g.,

Figure 4 of Japanese Patent Public Disclosure No. 2000-

342256). This system is a kind of binary vector system

because it consists of a disarmed Ti plasmid having the vir

5   region (virA, virB, virC, virD, virE and virG (hereinafter

sometimes each referred to as "vir fragment region")) and a

plasmid having the T-DNA. However, it differs in that it

uses a super-binary vector which a fragment of the vir

region substantially deprived of at least one of the vir

10   fragment regions (preferably the fragment including at

least virB or virG, more preferably the fragment including

virB and virG) is integrated, as the plasmid having the

T-DNA, i.e. a binary vector. Homologous recombination via

triparental mating can be used as a convenient method for

15   introducing the T-DNA region containing a desired gene into

Agrobacterium carrying a super-binary vector.

[0042] Agrobacterium that can be used as hosts in the

methods of the present invention are not specifically

limited, but preferably include *Agrobacterium tumefaciens*

20   (e.g., *Agrobacterium tumefaciens* LBA4404 (see Hoekema, A.,

et al., (1983), Nature, Vol.303, p.179-180) and EHA101 as

described above).

[0043] According to the methods of the present

invention, significant effects can be obtained, without

25   specific limitation, by using any gene transduction system

based on the expression of the genes in the virulence (vir)

region in Agrobacterium. Thus, benefits of the present

invention can be obtained by using any vector system such

as intermediate vectors, binary vectors, super-virulent
binary vectors and super-binary vectors as described above.
The same effects can also be obtained by using different
vector systems obtained by modifying these vectors (e.g.,
5   by excising a part or all of the vir region of
Agrobacterium and additionally inserting it into a plasmid,
or excising a part or all of the vir region and introducing
it as a part of a novel plasmid into Agrobacterium).
According to the methods of the present invention, the
10   infection efficiency can also be virtually improved with
wild-type Agrobacterium by increasing the introduction
efficiency of the wild-type T-DNA region into plants.

[0044]   A desired gene to be introduced into plants
can be inserted at a restriction endonuclease site in the
15   T-DNA region of the plasmid described above according to
standard procedures, and can be selected on the basis of a
suitable selective marker such as a gene having resistance
to a drug such as kanamycin or paromomycin simultaneously
or separately inserted into the plasmid. A desired DNA may
20   not be readily inserted into the T-DNA region of a large
plasmid having many restriction endonuclease sites by
conventional subcloning methods. In such cases, the
desired DNA can be inserted by homologous recombination in
cells of Agrobacterium via triparental mating. The size of
25   the transgene is not limited, but preferably about 100 bp
to 200 kbp.

[0045]   Introduction of the plasmid into an
Agrobacterium such as *Agrobacterium tumefaciens* can be

accomplished by conventional methods such as triparental mating as described above, electroporation, electroinjection, and chemical treatments with PEG or the like.

[0046] The gene to be introduced into plants is basically located between the left and right border sequences flanking the T-DNA in the same manner as in conventional techniques. However, only one border sequence may exist because the plasmid is circular, or three or more border sequences may exist when multiple genes are to be located at different sites. The gene may also be located on the Ti or Ri plasmid or on another plasmid in Agrobacterium. Alternatively, it may also be located on multiple types of plasmids.

[0047] <u>Step c)</u>

Agrobacterium-mediated gene introduction can be performed simply by contacting a plant material with an Agrobacterium. For example, it can be performed by preparing an Agrobacterium suspension having a cell density of about 10^6 to 10^{11} cells/ ml, immersing a plant material in this suspension for about 3 to 10 minutes, and then co-culturing them on a solid medium for several days.

[0048] Preferably, the plant material is co-cultured with Agrobacterium at the same time the plant material is infected with Agrobacterium or before Agrobacterium is removed after infection. Known media can be used for co-culture. For example, LS-AS medium and nN6-As medium used in the examples below or other media such as N6S3-AS

medium and 2N6-AS medium (see Hiei, Y., et al., (1994), The Plant Journal, Vol. 6, p. 271-282) are known.

[0049] In the present invention, the plant material may be subjected to at least one treatment selected from the group consisting of pressurization, heat treatment, centrifugation and sonication before or during step c) of infecting the plant material with an Agrobacterium. These treatments are also known to increase gene transduction efficiency in Agrobacterium-mediated gene transduction into plant materials. For example, centrifugation is described in documents (e.g., International Publication No. WO 02/12520; and Japanese Patent Public Disclosure No. 2000-342256), and preferably performed at 100 G to 250,000 G for 1 second to 4 hours. Heat treatment is described in documents (e.g., Japanese Patent Public Disclosure No. 2000-342255), and preferably performed in a temperature range of 33°C to 60°C for 5 seconds to 24 hours. Sonication is described in documents (e.g., Trick, H. N. and Finer, J. J., (1997), Transgenic Research, Vol.6, p.329-336; and Amoah, B., et al., (2001), Journal of Experimental Botany, Vol.52, p.1135-1142).

[0050] Any one of these treatments such as pressurization, heating, centrifugation and sonication may be performed or any combination may be performed. For example, Rogers, S. G., et al., (1988), Method for Plant Molecular Biology, p.423-436, CA: Academic Press Inc. describes a combination of heat treatment and centrifugation.

[0051] <u>Steps d) and e)</u>

In order to obtain a transformant if desired, step c)
described above should be followed by the steps of:

d) selecting a transformed cell; and

5   e) optionally regenerating the selected transformant. That
is, in order to perform transformation of a plant, it is
typically necessary to select a plant cell containing an
exogenous gene stably integrated into the chromosome after
the exogenous gene is introduced into the plant cell.

10   [0052] In the present invention, a medium enriched in
a metal salt containing copper ion may be used in step d)
of selecting a transformed cell, and/or step e) of
optionally regenerating the selected transformant.

[0053] The step of selecting a transformed cell means
15   selecting a cell having a desired trait based on phenotype
data and/or physical data.

[0054] Phenotype data such as transformation
efficiency can be obtained by evaluating the expression of
a marker gene and/or a selective marker gene co-introduced
20   with a gene desired to be introduced into a plant. Marker
genes and/or selective marker genes that can be used
include e.g., the GUS (β-glucuronidase) gene, antibiotic
resistance genes (e.g., PPT (phosphinothricin) resistance
genes, kanamycin resistance genes)), etc. When the GUS
25   gene is used as a marker gene, transformation efficiency
can be evaluated from the coloration resulting from the
cleavage of X-gulc (5-bromo-4-chloro-3-indolyl-β-D-
glucuronic acid) by GUS. When a gene resistant to an

antibiotic is used as a selective marker gene, evaluation can be made from the extent of growth on a selective medium containing the antibiotic after transformation.

[0055] To ascertain that the exogenous gene has been stably integrated into the chromosome, physical data such as southern blotting may be obtained. The selecting step may also be performed based on transmission to progeny via sexual reproduction and genetic and molecular analyses in progeny populations.

[0056] Optionally, the selected transformant may be regenerated and the regenerated plant may be grown to a whole plant. Regeneration from the selected transformant to a whole plant can be performed by known methods (e.g., Hiei, Y., et al., (1994), The Plant Journal, Vol. 6, p. 271-282; and Ishida, Y., et al., (1996), Nature Biotechnology, Vol.4, p.745-750).

[0057] The methods of the present invention improve gene introduction efficiency and/or transformation efficiency and/or promote the growth of regenerated plants as compared with cases where a medium containing a metal salt at a normal level is used. Gene introduction efficiency can be evaluated by e.g., assessing the area of the transient expression of the transgene. In the examples below, the transient expression of the GUS gene in the scutella of immature embryos was evaluated on a scale of five ratings from 1 (occasional spot expression) to 5 (expression on the entire surface of the scutellum). Alternatively, it can also be evaluated by counting the

total number of spots in cases where the total expression
level is low.

[0058] Transformation efficiency can be calculated by
e.g., counting the number of regenerated plants expressing
the GUS gene as transformants among those obtained from
inoculated immature embryos and dividing the total number
by the number of inoculated immature embryos.
Alternatively, it can also be calculated by counting the
number of regenerated plants showing resistance against a
selective pressure as transformants and dividing the total
number by the number of inoculated immature embryos.

[0059] The promotion of the growth of regenerated
plants can be evaluated by e.g., comparing the leaf length,
leaf area, and/or weight of regenerated plants obtained on
a medium enriched in a metal salt and a medium containing
the metal salt at a normal level.

The following examples further illustrate the present
invention without, however, limiting the invention thereto.
Those skilled in the art can readily add
modifications/changes to the present invention on the basis
of the description herein, and such modifications/changes
are included in the technical scope of the present
invention.

BRIEF DESCRIPTION OF THE DRAWING

[0060] [Figure 1] Figure 1 is a graph showing the
effects of adding copper sulfate to co-culture media on the
transient expression of the GUS gene in maize (A188). The

ordinate indicates quantification of the area of spots
showing the transient expression of the GUS gene in the
range from 0 (no expression) to 4 (expression on nearly the
entire surface of the scutellum). "Cu x" (where x
5   represents a number) shown on the abscissa of the graph
means that the concentration of copper sulfate in the co-
culture medium is x μM.

[Figure 2] Figure 2 is a graph showing the effects of
concentration of copper sulfate in co-culture media on
10   callus formation in maize (H99) after co-culture. On the
abscissa of the graph, "Ag x" and "Cu x" (where x
represents a number) mean that the concentrations of silver
nitrate and copper sulfate in the co-culture medium are x
μM, respectively.

15   [Figure 3] Figure 3 is a graph showing the effects of
concentration of copper sulfate in co-culture media on
phosphinothricin (PPT)-resistant callus formation in maize
(A188) after co-culture. The ordinate indicates
quantification of callus formation at levels from 0 (no
20   callus formed) to 3 (the entire scutellum formed callus).
"Cu x" (where x represents a number) shown on the abscissa
of the graph means that the concentration of copper sulfate
in the co-culture medium is x μM.

[Figure 4] Figure 4 is a graph showing the effects of
25   adding copper sulfate to a coculture medium on the
transient expression of the GUS gene in rice (IR64). The
ordinate indicates quantification of the area of spots
showing the transient expression of the GUS gene in the

range from 0 (no expression) to 4 (expression on nearly the entire surface of the scutellum). "Cu 5" shown on the abscissa of the graph means that the concentration of copper sulfate in the co-culture medium is 5 μM.

5   [Figure 5] Figure 5 is a graph showing the effects of adding copper gluconate to a co-culture media on the transient expression of the GUS gene in maize (A188). The ordinate indicates quantification of the area of spots showing the transient expression of the GUS gene in the

10   range from 0 (no expression) to 4 (expression on nearly the entire surface of the scutellum).

[Figure 6] Figure 6 is a graph showing the effects of adding copper gluconate to co-culture media on callus formation in maize (A188). The ordinate indicates

15   quantification of callus formation at levels from 0 (no callus formed) to 3 (the entire scutellum formed callus).

[Figure 7] Figure 7 is a graph showing the effects of adding copper sulfate or copper gluconate to co-culture media on the growth of immature embryos of rice

20   (Yukihikari). The ordinate indicates the average weight of immature embryos which formed callus, per immature embryo. On the abscissa, Experiments 1, 2, 3 represent experiments independently performed; cont. represents a control lacking copper sulfate or copper gluconate; Cu represents copper

25   sulfate; and CG represents copper gluconate. The numerical values following Cu and CG represent their concentrations (μM) in the coculture medium.

[Figure 8] Figure 8 is a graph showing the effects of

adding copper sulfate or copper gluconate to regeneration media on the growth of transformed plants regenerated from maize (A188) transformed calli. The ordinate indicates the average of the leaf length of regenerated plants. On the abscissa, cont. represents a control lacking copper sulfate or copper gluconate; Cu represents copper sulfate; and CG represents copper gluconate. Copper sulfate and copper gluconate were added at 10 μM to their co-culture media.

[Figure 9] Figure 9 is a graph showing the effects of adding copper sulfate or copper gluconate to regeneration media on the growth of transformed plants regenerated from rice (Yukihikari) transformed calli. The ordinate indicates the average of the leaf length of regenerated plants. On the abscissa, cont. represents a control lacking copper sulfate or copper gluconate; Cu represents copper sulfate; and CG represents copper gluconate. Copper sulfate and copper gluconate were added at 10 μM to their co-culture media.

EXAMPLES

[0061]   Example 1: Effects of adding copper sulfate to co-culture media on maize transformation

Materials and Methods

Immature embryos (size: 1.0-1.5 mm) of maize (cultivars: A188, H99) at days 7-14 post-pollination were aseptically collected and washed once in LS-inf liquid medium (LS salts, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine hydrochloride, 1 mg/l thiamine hydrochloride, 100 mg/l

myoinositol, 1 g/l casamino acids, 1.5 mg/l 2,4-D, 68.5 g/l
sucrose, 36 g/l glucose, pH 5.2; see Ishida, Y., et al.,
(1996), Nature Biotechnology, Vol.4, p.745-750). The
immature embryos were partially pretreated for increasing

5 the gene transduction efficiency (heated at 46°C for
3 minutes and centrifuged at 15,000 rpm for 10 minutes).
Agrobacterium tumefaciens LBA4404 (pSB131) (carrying a PPT
(phosphinothricin) resistance gene driven by the
cauliflower mosaic virus 35S promoter and the GUS gene

10 containing a castor bean catalase intron fused to the
cauliflower mosaic virus 35S promoter in the T-DNA region;
see Ishida, Y., et al., (1996), Nature Biotechnology,
Vol. 4, p. 745-750) was suspended at about 1.0×10^9 cfu/ml
in LS-inf liquid medium containing 100 µM acetosyringone to

15 prepare an inoculum. The inoculum was added to the
collected/washed immature embryos and heated/centrifuged
immature embryos, and the suspensions were agitated for
30 seconds and then allowed to stand for 5 minutes at room
temperature. The Agrobacterium-inoculated immature embryos

20 were plated on co-culture media containing $CuSO_4 \cdot 5H_2O$ at a
concentration of 0-10 µM in LS-AS medium containing 5 µM
$AgNO_3$ (LS salts, 0.5 mg/l nicotinic acid, 0.5 mg/l
pyridoxine hydrochloride, 1 mg/l thiamine hydrochloride,
100 mg/l myoinositol, 700 mg/l L-proline, 1.5 mg/l 2,4-D,

25 20 g/l sucrose, 10 g/l glucose, 500 mg/l MES, 100 µM
acetosyringone, 8 g/l agar, pH 5.8; see Ishida, Y., et al.,
(1996), Nature Biotechnology, Vol. 4, p. 745-750, in which
8 g/l agarose is used as a gelling agent) with the

embryonic disc facing upward.

[0062] The immature embryos were incubated at 25°C in darkness for 3 days and then partially immersed in a 0.1 M phosphate buffer (pH 6.8) containing 0.1% Triton X-100, and allowed to stand at 37°C for 1 hour. After Agrobacterium was removed with the phosphate buffer, a phosphate buffer containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) and 20% methanol was added. The immature embryos were treated at 37°C for 24 hours, and then observed under a microscope to assess the area of blue-stained tissue.

[0063] The immature embryos incubated on co-culture media for 3 days were plated on LSD 1.5 medium (LS salts, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine hydrochloride, 1 mg/l thiamine hydrochloride, 100 mg/l myoinositol, 700 mg/l L-proline, 1.5 mg/l 2,4-D, 20 g/l sucrose, 500 mg/l MES, 8 g/l agar, pH 5.8; see Ishida, Y., et al., (1996), Nature Biotechnology, Vol. 4, p. 745-750) and incubated at 25°C in darkness for about 4 weeks, and then measured for the diameters of grown calli. The immature embryos incubated on co-culture media for 7 days were seeded on LSD 1.5 medium containing 5 mg/l phosphinothricin (PPT) and incubated at 25°C in darkness for 1 week, and then observed under a microscope to assess the extent of callus formation. These calli were cultured on the same medium containing 10 mg/l PPT for 6 weeks under the same conditions. Grown PPT-resistant calli were seeded on LSZ regeneration medium (LSD 1.5 medium deprived of 2,4-D and

supplemented with 5 mg/l zeatin) and incubated at 25°C

under illumination for 2-3 weeks. Leaf segments of

regenerated plants were excised and assessed for the

expression of the GUS gene by X-gluc.

5   [0064] Results

Immature embryos (cultivar: A188) incubated on various

coculture media for 3 days were stained with X-gluc, and

the area of spots showing the transient expression of the

GUS gene (blue spots) was evaluated on a scale of five

10   ratings from 0 (no expression) to 4 (expression on nearly

the entire surface of the scutellum).

[0065] Immature embryos incubated on co-culture media

containing 1, 5 and 10 μM copper sulfate for 3 days showed

the transient expression of the GUS gene in a wider area of

15   the scutellum as compared with immature embryos incubated

on a control medium. The increase of the area showing the

transient expression of the GUS gene by adding copper

sulfate to co-culture media was observed irrespective of

whether or not heat/centrifugation pretreatment was

20   performed (Figure 1). These results showed that the gene

introduction efficiency increases by adding copper sulfate

to co-culture media.

[0066] Immature embryos (cultivar: H99) incubated on

various co-culture media for 3 days were cultured on a

25   medium containing no selective pressure for about 4 weeks,

and then measured for the diameters of formed calli.

Immature embryos incubated on a co-culture medium

containing 5 μM silver nitrate showed callus growth nearly

comparable to that of immature embryos cultured on a control medium. In contrast, immature embryos incubated on a co-culture medium containing 10 μM copper sulfate and 5 μM silver nitrate had an average diameter greater than

5 that of immature embryos incubated on a control co-culture medium by 2 mm or more, showing that addition of copper sulfate promotes callus growth (Figure 2).

[0067] After co-culture for 1 week, the calli grown from immature embryos (cultivar: A188) cultured on a

10 selective medium containing PPT for 1 week were evaluated on a scale of four ratings from 0 (no callus formed) to 3 (the entire scutellum formed callus). Immature embryos cultured on media containing copper sulfate showed higher callus formation as compared with immature embryos cultured

15 on a control medium, showing that addition of copper sulfate improves the efficiency of transformed callus formation (Figure 3). Further selection on a medium containing PPT and incubation of the resulting calli in a regeneration medium containing PPT gave PPT-resistant

20 plants. Leaf segments of these plants were excised and assessed for the expression of the GUS gene. As a result, immature embryos incubated on co-culture media containing 5 and 10 μM copper sulfate showed a 2- to 3-fold higher transformation efficiency than that of immature embryos

25 incubated on a co-culture medium lacking copper sulfate (Table 1).

[0068]

Table 1. Effects of adding copper sulfate to co-culture
media on transformation efficiency

CuSO$_4$ (μM)	Number of inoculated immature embryos (A)	Number of regenerated plants	Number of GUS-positive plants (B)	Transformation efficiency (B/A,%)
0	13	2	2	15.4
1	13	2	2	15.4
5	13	6	6	46.2
10	14	6	5	35.7

Thus, it was shown that addition of copper sulfate to

5   co-culture media has the effect of increasing

Agrobacterium-mediated gene introduction efficiency,

promoting callus formation and growth, and improving

transformation efficiency.

10   Example 2: Effects of adding copper sulfate to co-

culture media on gene introduction of rice

Materials and Methods

An *Agrobacterium tumefaciens* super-binary vector

designated LBA4404 (pSB134) (carrying the HPT gene

15   (hygromycin resistance gene) fused to a ubiquitin intron

driven by a maize ubiquitin promoter and the GUS gene fused

to a castor bean catalase intron driven by the cauliflower

mosaic virus 35S promoter in the T-DNA region; construction

of pSB134 was performed by inserting a 35S-intron GUS-nos

20   fragment derived from pSB32 as an expression marker into

HindIII of pKY205 (see WO 03/027290)) cultured on AB medium

(3 g/l KH_2PO_4, 1 g/l NaH_2PO_4, 1 g/l NH_4Cl, 300 mg/l $MgSO_4\cdot$

$7H_2O$, 150 mg/l KCl, 10 mg/l $CaCl_2$, 2.5 mg/l $FeSO_4\cdot7H_2O$,

5 g/l glucose, 15 g/l agar, pH 7.0; Chilton, M.-D., et al.,

(1974), Proc. Natl. Acad. Sci. U.S.A., 71:3672-3676)

5   containing 50 mg/l hygromycin and 50 mg/l spectinomycin for

3 to 4 days was scraped with a platinum loop and suspended

at a concentration of about 10^9 cfu/ml in 1 ml of AA1

liquid medium (AA major salts, LS minor salts, MS vitamins,

AA amino acids, 0.2 g/l casamino acids, 4 g/l sucrose,

10 2 g/l glucose, pH 5.2) containing 100 μM acetosyringone.

Into an Eppendorf tube containing aseptically collected

immature embryos (cultivar: IR64) was added 1 ml of the

Agrobacterium suspension and the tube was agitated for

30 seconds in a vortex mixer and then allowed to stand at

15 room temperature for 5 minutes. The immature embryos were

plated on nN6-As medium (N6 salts, N6 vitamins, 0.5 g/l

casamino acids, 0.5 g/l L-proline, 1 mg/l 2,4-D, 0.5 mg/l

NAA, 0.1 mg/l 6BA, 20 g/l sucrose, 10 g/l glucose, 10 μM

$AgNO_3$, 100 μM acetosyringone, 8 g/l agarose, pH 5.2) and

20 nN6-As medium containing 5 μM $CuSO_4\cdot5H_2O$ and incubated in

darkness at 25°C for one week.

[0069]   Results

Co-cultured immature embryos were stained with X-gluc,

and the area of spots showing the transient expression of

25 the GUS gene (blue spots) was evaluated on a scale of five

ratings from 0 (no expression) to 4 (expression on nearly

the entire surface of the scutellum).

[0070]   Immature embryos incubated on a co-culture

medium containing 5 µM copper sulfate for 1 week showed the transient expression of the GUS gene in a wider area of the scutellum as compared with immature embryos cultured on a control medium (Figure 4). This showed that the

5 improvement in gene introduction efficiency by adding copper sulfate to co-culture media is found in not only maize but also rice.

Example 3: Effects of adding copper gluconate to

10 co-culture media on gene introduction of maize

Materials and Methods

Immature embryos (size: 1.0-1.5 mm) of maize (cultivar: A188) at days 7-14 post-pollination were aseptically collected and washed once in LS-inf liquid medium. A

15 pretreatment for increasing gene transduction efficiency (heating at 46°C for 3 minutes and centrifugation at 15,000 rpm for 10 minutes) was performed. *Agrobacterium tumefaciens* LBA4404 (pSB131) was suspended at about 1.0×10^9 cfu/ml in LS-inf liquid medium containing 100 µM

20 acetosyringone to prepare an inoculum. The inoculum was added to the heated/centrifuged immature embryos, and the suspension was agitated for 30 seconds and then allowed to stand for 5 minutes at room temperature. The Agrobacterium-inoculated immature embryos were plated on

25 co-culture media containing copper gluconate at a concentration of 0-10 µM in LS-AS medium containing 5 µM AgNO$_3$ with the scutellum facing upward.

[0071] The immature embryos were incubated at 25°C in

darkness for 3 days and then partially immersed in a 0.1 M

phosphate buffer (pH 6.8) containing 0.1% Triton X-100, and

allowed to stand at 37°C for 1 hour. After Agrobacterium

was removed with the phosphate buffer, a phosphate buffer

5   containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic

acid (X-gluc) and 20% methanol was added. The immature

embryos were treated at 37°C for 24 hours, and then

observed under a microscope to assess the area of blue-

stained tissue.

10   [0072] The immature embryos incubated on co-culture

media for 7 days were evaluated for callus formation on a

scale of ratings 0 (no callus formed), 1 (a part of the

scutellum formed callus), 2 (about a half of the scutellum

formed callus), and 3 (3/4 or more of the scutellum formed

15   callus).

[0073] Results

Immature embryos (cultivar: A188) incubated on various

co-culture media for 3 days were stained with X-gluc, and

the area of spots showing the transient expression of the

20   GUS gene (blue spots) was evaluated on a scale of five

ratings from 0 (no expression) to 4 (expression on nearly

the entire surface of the scutellum). Immature embryos

incubated on co-culture media containing 1, 5 and 10 μM

copper gluconate for 3 days showed the transient expression

25   of the GUS gene in a wider area of the scutellum as

compared with immature embryos cultured on a control medium

(Figure 5).

[0074] Immature embryos incubated on co-culture media

for 1 week was assessed for callus formation. All immature
embryos incubated on co-culture media containing 1, 5 and
10 µM copper gluconate showed higher callus formation than
that of immature embryos co-cultured on a medium lacking
5   copper gluconate. Especially, significantly higher callus
formation was shown on co-culture media containing 5 and
10 µM copper gluconate as compared with control (Figure 6).

[0075] These results showed that gene introduction
efficiency and callus formation efficiency increase by
10   adding copper gluconate to co-culture media in the same
manner that copper sulfate is added.

Example 4: Effects of adding copper sulfate and copper
gluconate to inoculum liquid media on gene introduction of
15   rice.

Materials and Methods

An *Agrobacterium tumefaciens* super-binary vector
designated LBA4404 (pSB134) cultured on AB medium
containing 50 mg/l hygromycin and 50 mg/l spectinomycin for
20   3 to 4 days was scraped with a platinum loop and suspended
at a concentration of about 10^9 cfu/ml in 1 ml of AA1
liquid medium containing 100 µM acetosyringone and 0-50 µM
$CuSO_4 \cdot 5H_2O$ or copper gluconate. Into an Eppendorf tube
containing aseptically collected immature embryos
25   (cultivar: Yukihikari) was added 1 ml of the Agrobacterium
suspension and the tube was agitated for 30 seconds in a
vortex mixer and then allowed to stand at room temperature
for 5 minutes. The immature embryos were seeded on nN6-As

medium and incubated in darkness at 25°C for one week.

[0076] Results

The weight of co-cultured immature embryos was
measured. In all three experiments, immature embryos
5 cultured on inoculum liquid media containing copper sulfate
or copper gluconate showed more active growth than immature
embryos cultured on a control medium lacking copper sulfate
or copper gluconate (Figure 7). This showed that the
improvement in callus growth by adding copper sulfate and
10 copper gluconate is equally observed not only when they are
added to co-culture media but also when they are added to
inoculum liquid media.

Example 5: Effects of adding copper sulfate, copper
15 gluconate to regeneration media on the growth of
transformed maize plants.

Materials and Methods

Immature embryos (size: 1.0-1.5 mm) of maize
(cultivar: A188) were inoculated with *Agrobacterium*
20 *tumefaciens* LBA4404 (pSB131) and cultured on LSD 1.5 medium
containing PPT to give a transformed callus. The
transformed callus was cut into a size of about 2 mm and
plated on LSZ regeneration medium containing 10 μM $CuSO_4 \cdot$
$5H_2O$ or copper gluconate, and PPT. After incubation at 25°C
25 under illumination for 3 weeks, the leaf length of
regenerated plants was measured.

[0077] Results

The leaf length of plants regenerated on a

regeneration medium containing copper sulfate or copper

gluconate was significantly longer than that of plants

regenerated on a control regeneration medium, showing that

copper sulfate or copper gluconate has the effect of

5   promoting the growth of regenerated plants (Figure 8).

Example 6: Effects of adding copper sulfate, copper

gluconate to regeneration media on the growth of

transformed rice plants.

10   Materials and Methods

Immature embryos of rice (cultivar: Yukihikari) were

inoculated with *Agrobacterium tumefaciens* LBA4404 (pSB134)

and cultured on nN6CC medium (N6 salts, N6 vitamins, 0.5

g/l casamino acids, 0.5 g/l L-proline, 1 mg/l 2,4-D, 0.5

15   mg/l NAA, 0.1 mg/l 6BA, 20 g/l sucrose, 55 g/l sorbitol,

250 mg/l cefotaxime, 250 mg/l carbenicillin, 5 g/l Gelrite,

pH 5.8) containing hygromycin to give a transformed callus.

The transformed callus was cut into a size of about 2 mm

and seeded on N6R regeneration medium (N6 salts with major

20   salts reduced to 1/2, N6 vitamins, AA amino acids, 1 g/l

casamino acids, 0.5 mg/l kinetin, 20 g/l sucrose, 30 g/l

sorbitol, 4 g/l Gelrite, pH 5.8) containing 10 μM CuSO$_4$·5H$_2$O

or copper gluconate, and hygromycin. After incubation at

25°C under illumination for 3 weeks, the leaf length of

25   regenerated plants was measured.

[0078] Results

The leaf length of plants regenerated on a

regeneration medium containing copper sulfate or copper

gluconate was significantly longer than that of plants regenerated on a control regeneration medium, whereby it was also shown in rice that copper sulfate or copper gluconate has the effect of promoting the growth of

5 regenerated plants (Figure 9).

INDUSTRIAL APPLICABILITY

[0079] The present invention provides inexpensive and convenient gene introduction methods with higher efficiency

10 than conventional Agrobacterium-mediated methods. It also provides methods that can be adapted to plant species and cultivars involving difficulty in gene introduction by conventional Agrobacterium-mediated methods. The methods of the present invention improve gene introduction

15 efficiency and/or transformation efficiency and/or promote the growth of regenerated plants as compared with cases where a medium containing a metal salt at a normal level is used.

[0080] As shown in Figure 1, the gene introduction

20 efficiency in a monocotyledon maize was improved to a 2-fold to 2.5-fold higher level by using co-culture media containing copper sulfate at high concentrations as compared with the case where a medium lacking copper sulfate was used. The gene introduction efficiency was

25 further improved to a 1.5-fold to 3-fold higher level by heating/centrifugation as compared with the case where a medium lacking copper sulfate and untreated plant materials were used.

[0081] The present invention improved Agrobacterium-mediated gene introduction efficiency in plants, thereby allowing many transformed plants to be efficiently obtained, contributing to efficient and easy culture of cultivars containing a practical gene. Especially, the improvement in transformation efficiency by the methods of the present invention is very significant because the transformation efficiency of monocotyledons, especially maize by conventional Agrobacterium-mediated methods is low.